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In the rd mouse the absence of rod cells and the progressive loss of cones does not result in a decrease in circadian phase shifting responses to light. By contrast, rd mice are unable to perform simple visual tasks. In addition, rodless transgenic mice, and mice homozygous for the rds mutation, show unattenuated circadian responses to light. Collectively these data suggest that cone cells lacking outer segments are sufficient to maintain normal circadian responses to light, or there may be some unidentified photoreceptor withi the retina. An action spectrum for circadian responses to light in rd m..., and molecular analysis of retinally degenerate mice and blind mole rat eyes, suggests the involvement of a green cone opsin in mammalian photoentrainment.

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Summary: In the *rd* mouse the absence of rod cells and the progressive loss of cones does not result in a decrease in circadian phase shifting responses to light. By contrast, *rd* mice are unable to perform simple visual tasks. In addition, rodless transgenic mice, and mice homozygous for the *rds* mutation, show unattenuated circadian responses to light. Collectively these data suggest that cone cells lacking outer segments are sufficient to maintain normal circadian responses to light, or there may be some unidentified photoreceptor within the retina. An action spectrum for circadian responses to light in *rd* mice, and molecular analysis of retinally degenerate mice and blind mole rat eyes, suggests the involvement of a green cone opsin in mammalian photoentrainment.

Introduction

Daily or circadian rhythms are driven by endogenous pacemakers that have periods close to, but rarely exactly, 24 hours. In order to function adaptively, circadian systems must be synchronized (entrained) with the astronomical day, and for the majority of circadian rhythms internal time is entrained by the irradiance changes at dawn and dusk. While it is clear that photoreceptive mechanisms are used to regulate the clock, our understanding of these sensory systems remains superficial. The nature of the photopigment and even the location of the photoreceptor cells remain unknown.

In the vertebrates, all photoreceptors appear to arise from the diencephalon, and are found within the retina, pineal complex (pineal, parapineal, parietal, frontal organ) and "deep brain" [[17]]. Despite this common origin, these photoreceptors differ markedly and have been broadly grouped into two classes: visual or retinal photoreceptors which mediate image detection, and extraretinal photoreceptors which detect irradiance changes and regulate circadian and other autonomic physiological responses to light. In mammals this distinction is blurred. Mammals lack extraretinal photoreceptors, but have unidentified photoreceptors within the eye that regulate circadian responses to light [[9]].

Circadian Photoreception in Mammals

We have previously shown that circadian responses to light are unaffected in mice homozygous for the *retinal degeneration* mutation (*rd/rd*) [[9]]. This mutation results in the rapid loss of rods followed by the more protracted loss of cones [[3, 6]]. The remaining cone cells appear to lack outer segments. Despite this tremendous loss in visual machinery, the site of circadian photoreception still resides within the degenerate eye because bilateral enucleation of *rd* animals abolishes all circadian responses to light. These data suggest that circadian photoreception is either normally performed by small numbers of cone photoreceptors (which lack outer segments), or alternatively, there may exist an unrecognized class of photoreceptive cell in the mammalian retina that is unaffected by the *rd* mutation and that functions normally to regulate rhythmic physiology and behavior [[9]]. In this report I will review our most recent findings and address these alternatives.

(a) Circadian and Visual Responses in Aged Retinally Degenerate Mice. The progression of photoreceptor degeneration in *rd* mice commences early in post-natal development with the loss of rods, followed by a more protracted loss of cones. Our original studies examined circadian responses to light in *rd* and normal mice 80 days of age, and we found that phase shifting responses were identical. If the surviving cone cell bodies mediate circadian responses to light, and assuming that the circadian system uses some photon counting mechanism (Section (e)), then we would expect the photosensitivity of the circadian system to decline with age and parallel the loss of cones in the *rd* retina. We have examined circadian photosensitivity in aged *rd* and normal mice (80 - 800 days of age) and find no attenuation of response. Thus, there appears to be no correlation between circadian responses to light and rod or cone cell loss in the *rd* retina [[2, 19]].

In parallel with our analysis of circadian responses to light, we have developed behavioral assays to assess the visual capabilities of retinally degenerate mice. A mouse is placed in a two-chambered cage with an escape window placed in the center of the dividing wall through which the mouse can move freely. The mouse can be given a mild electrical shock (0.25mA) through the wire floor of either side of the cage, but never both chambers at the same time, allowing the animal to escape to the neutral chamber when the shock is applied. For the first two days of testing, the mouse is tested in dim red light and given a 10 second shock every 60 seconds for 20 trials. The mouse learns to escape the shock only by jumping through the window to the neutral side of the cage. For the following 14 days, a 5 second white light pulse (25 $\mu W/cm^2$) is administered prior to the electrical shock. Escape to the neutral chamber and hence avoidance of the shock demonstrates visual competence. Normal mice successfully learn to avoid the shock, but *rd* mice seem incapable of learning this task. However, the substitution of a 3kHz tone for the light pulse resulted in classical conditioning of both normal and *rd* animals [[19]]. Recall that light-induced circadian responses of *rd* mice remain unattenuated, yet these animals fail to respond to this simple visual task. These data illustrate major differences between visual and circadian light detection.

(b) Rod photoreceptors are not required for circadian responses to light. Circadian responses to light were investigated in transgenic mice carrying an integrated fusion gene consisting of a 1 kb fragment of the human rhodopsin promoter linked to the attenuated diphtheria toxin (DT-A) gene. Morphological analysis of the retinae demonstrates that rod photoreceptors are eliminated, but cone cell bodies (with no outer segments) remain for at least 11 weeks after birth [[15]]. Despite this loss, circadian locomotor rhythms entrain to a light:dark cycle, and freerunning rhythms in constant darkness can be phase shifted by light in a manner indistinguishable from normal mice (**Figure 1**) [[8]]. These data support our results in *rd* mice and demonstrate that rod photoreceptors are not required for circadian responses to light.

(c) Photoreceptor outer segments are not required for circadian responses to light. Another retinal mutation, *rds* (*retinal degeneration slow*), in the C3H mouse strain has provided an additional approach to the question of which elements in the eye mediate circadian responses to light. In *rds* mice, rod and cone photoreceptors fail to develop outer segments and as a result both classes of photoreceptors gradually degenerate over the course of a year [[1, 20]] (*rd*

.ptors develop outer segments but are lost as retinal degeneration proceeds). In *rds* ately half of all photoreceptor cell bodies have degenerated by three months, and e gone by one year of age. Our studies have shown that circadian responses to light . in *rds*, *rd* and +/+ genotypes (**Figure 2**), even in aged animals [[2, 19]]. In addition ıg our conviction that only a small population of photoreceptor cells are required for the . system to entrain, these data also provide overwhelming evidence that outer segments are uired to elicit a circadian response to light.

(d) The spectral sensitivity of circadian responses to light resemble green cones. We have now completed an action spectrum for phase-shifting circadian rhythms in *rd* and +/+ mice. From a series of irradiance response curves at six different wavelengths, we derived an action spectrum that closely fits a visual pigment nomogram with a wavelength of maximum sensitivity near 505 nm (Provencio & Foster, in preparation). Because rods are not required for circadian responses to light (Sections (a) & (b)), then cones become obvious candidates for circadian photoreception. To date, there is evidence for two types of cones within the mouse retina. Electroretinogram (ERG) and behavioral studies have shown two sensitivity maxima, a green-sensitive cone near 510 nm and an ultraviolet sensitive cone near 370 nm [[10]]. The similarity of our action spectrum results (505 nm) to the spectral sensitivity of the green cones (510 nm) suggests that the green cones, or green cone opsin, may mediate photoentrainment in mice.

(e) Molecular analysis of the remaining opsins within the *rd* eye. To date, only three opsins have been cloned from the mouse retina (Dr. M. Applebury, personal communication). On the basis of their similarity to human opsins and ERG responses recorded from the mouse eye they can be classified as: rod opsin, UV/blue cone opsin, and green cone opsin. In an attempt to implicate these opsins in circadian responses to light, we initially performed Northern blot analysis to quantify the loss of these opsins from the degenerating (*rd*) mouse retina. Rod opsin mRNA disappeared by four weeks of age, followed by the gradual loss of UV and green cone opsins to below detectable levels by 45 weeks of age [[2]]. These data suggest two broad alternatives: either one or more of the known opsins mediates circadian responses to light and occurs at extremely low levels in the degenerate retina, or none of the known opsins mediate circadian responses to light, and there is a unique "circadian" opsin in the retina.

To achieve greater sensitivity we have extended our molecular characterization by using RT-PCR (reverse transcriptase - polymerase chain reaction). We have succeeded in amplifying both the green cone and UV cone opsin, but failed to identify any rod opsin mRNA in the *rd* retina over one year of age. As the two cone opsin messages remain, both proteins could play a role in mediating circadian responses to light. Our action spectrum results suggest the involvement of the green cone opsin (Section 2d), but as the action spectrum did not explore the effects of wavelengths in the UV range, we cannot dismiss the involvement a UV opsin in photoentrainment.

Arguments for the involvement of green cones in circadian regulation would be strengthened if we could show that these cells are connected to the suprachiasmatic nuclei, the primary site of the mammalian circadian pacemaker. We are currently using the Bartha strain of

porcine pseudorabies virus (generously donated by J.P. Card, University of Pittsburgh) for tract tracing studies on the mouse retinohypothalamic tract. The infective properties of this virus are "circadian system-specific"[[4]]. When injected into the eye of an *rd* or normal mouse the virus labels the suprachiasmatic nuclei (SCN), but in the initial wave of the infection leaves visual projections uninfected [[18]]. This technique offers us the possibility of tracing the connections between defined retinal cells and clock centers within the brain (SCN).

It is worth stressing that although green cone opsins and photoreceptor cell bodies remain in the *rd* retina, most of the green cones are lost from the retina. If the green sensitive cones (lacking outer segments) mediate circadian responses to light, then one must explain the observation that circadian responses to light remain unattenuated in aged *rd* mice. We recently proposed a model that could account for loss of photoreceptors with no loss in sensitivity [[7]]. This model can be summarized as follows: if circadian photosensitivity were directly related to photoreceptor number (i.e. photons are counted by an additive process), then one would expect to observe a decline in circadian responses to light as photoreceptors disappeared from the retina. If, however, the output from circadian photoreceptors is averaged, then a progressive loss of circadian sensitivity would not be observed until the number of photoreceptor cells approached zero. Such a system would require relatively few photoreceptive cells to show normal responses to light. In this case the sensitivity-limiting step is "down stream" from the photoreceptors and the system would be buffered from the degenerative loss of photoreceptive elements.

(f) Analysis of a blind subterranean mammal: The blind mole rat (*Spalax ehrenberghi*). The blind mole rat, *Spalax ehrenberghi*, is a subterranean mammal whose visual system is naturally drastically reduced. These animals have minute subcutaneous eyes that lack a functional lens. As a result the eye lacks any image forming capability. In addition, projections to visual centers of the brain are reduced by 87-93% when compared with other rodents. By contrast, the relative size and morphology of the suprachiasmatic nuclei (SCN), in which the circadian pacemaker resides, is identical between *Spalax* and other rodents. Moreover, the retinal projections to the SCN in the mole rat are increased twenty-fold [[5]]. We have recently shown, using viral tract tracing techniques, that all the ganglion cells within the retina project to the SCN (Cooper, Provencio, Foster unpublished data). Although visual responses are lacking in *Spalax*, photic entrainment of locomotor and thermoregulatory activity rhythms persist. With these characteristics, these eyes have been considered to be "pure" circadian photoreceptors. Using primers for the three mouse opsins (rod, blue/UV cone and red/green cone) and a pair of degenerate universal cone primers, we conducted RT-PCR on RNA isolated from blind mole rat eyes in an attempt to PCR-clone any opsins present in the eye. Only the mouse red/green primers produced an amplification product. This cDNA shows high sequence homology to both the mouse and human green cone opsins (**Figure 3**). These preliminary data in the mole rat would support our *rd* data and the proposal that a green cone opsin plays a major role in the regulation of mammalian circadian responses to light.

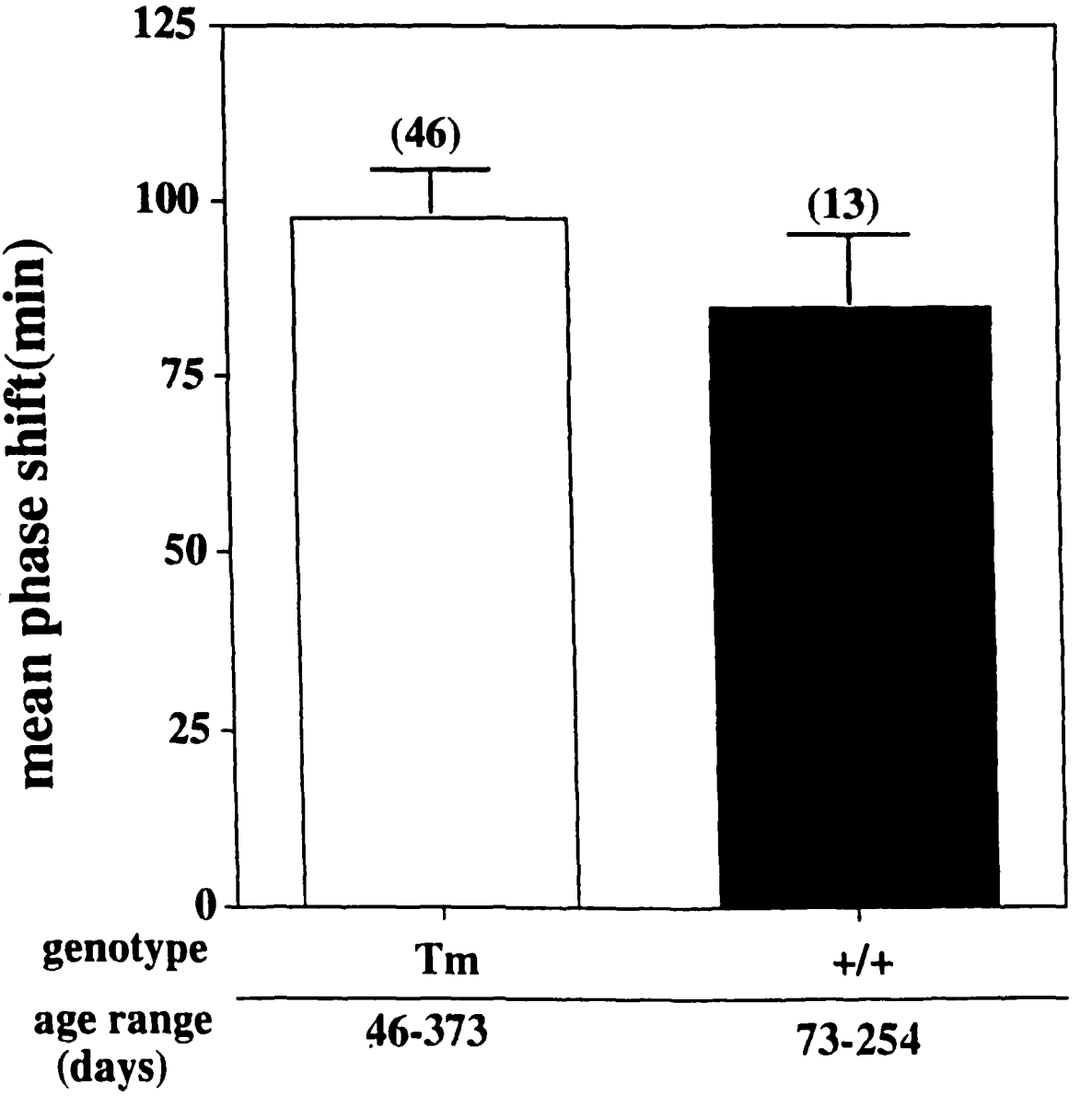
Conclusions: The photoreceptors that regulate circadian responses in mammals remain unidentified. Mice (*rd*, *rds*, rodless transgenics) in which visual responses are lacking show unattenuated circadian responses to light, demonstrating that the organization of visual and circadian light detection is quite different. Rod photoreceptors are not required, and if cone cells mediate circadian responses to light, then relatively few cells (lacking outer segments) are sufficient to maintain normal sensitivities. Of the known opsins in the mouse retina, the green cone opsin is the strongest candidate for photoentrainment, although we cannot currently preclude any involvement of the UV opsin. In the blind mole rat, an animal with subcutaneous rudimentary eyes, lacking visual responses but showing photoentrainment, we find evidence for an opsin that is almost identical to the mouse and human green cone opsins. Whether mammalian circadian responses to light are mediated by cones themselves or by a cone opsin in some unidentified retinal cell type remains to be determined. In addition, we cannot preclude the possibility that some novel "circadian" opsin exists within the retina.

Fig. 1. Histogram representing mean phase shifting responses of C57BL wild-type (+/+) and rodless transgenic (Tm) mice of various ages. Transgenic mice carry a DT-A gene under the control of the human rod opsin promoter. Phase shifting paradigm is identical to description in legend for Fig. 2. Sample sizes are indicated in parenthesis. Error bars represent the standard error of the mean.

Fig. 2. Free-running locomotor activity rhythms of C3H +/+ **(A, n=48)**, *rd/rd* **(B, n=54)** and *rds/rds* **(C, n=45)** mice housed in complete darkness in cages equipped with running wheels. Each record is plotted on a 24 hour scale with subsequent days plotted from top to bottom. Black bars represent bouts of concentrated wheel-running activity. After 10-12 days in constant darkness, the mice were individually placed into a light pulse apparatus and exposed to a 15 minute pulse of monochromatic light (515nm; $1X10^{-1}\mu W/cm^2$) at circadian time 16 (CT16), four hours after activity onset (CT12). The time of the pulse is represented by a filled circle. After an additional 10 days in darkness, the magnitude of the phase shift was measured as the difference between the steady state phase of the free-running rhythm before and after the pulse (Δ).

Fig. 3. Partial amino acid sequence of the *Spalax* clone and other known middle-wavelength sensitive cone opsins. The clone spans the region between Helix II and Helix III of the opsin molecule. Sequences with 100% identity between species are boxed. Mouse opsin sequence (a) has been determined by Dr. M. Applebury (personal communication). Other red/green sequences (b-f) have previously been reported by Nathans et al. ([16]), Kojima et al. ([13]), Kuwata et al. ([14]), Kawamura and Yokoyama ([12]), and Johnson et al. ([11]), respectively.

Figure 1


125
100
75
50
25
0
mean phase shift(min)
(46)
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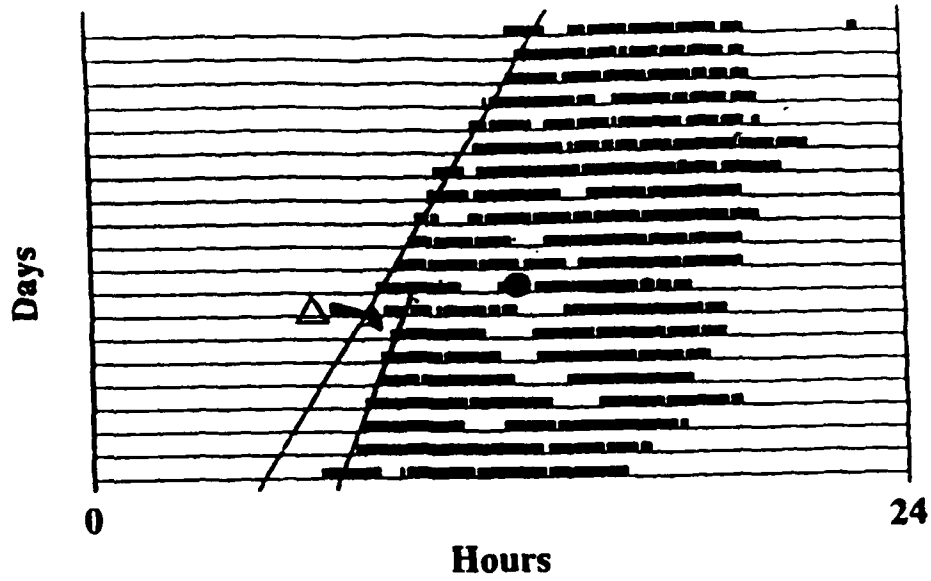
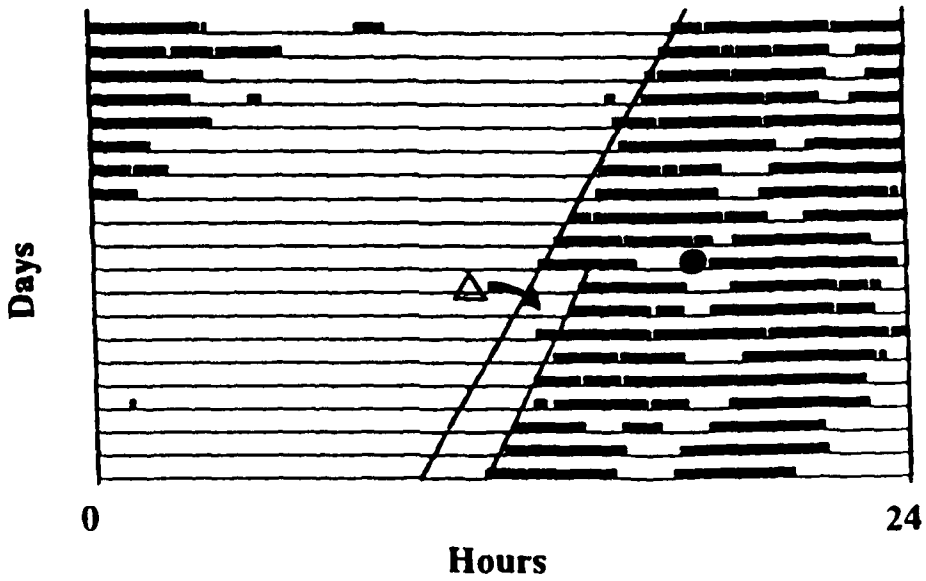
genotype
Tm
+/+
age range
(days)
46-373
73-254

Fig 2

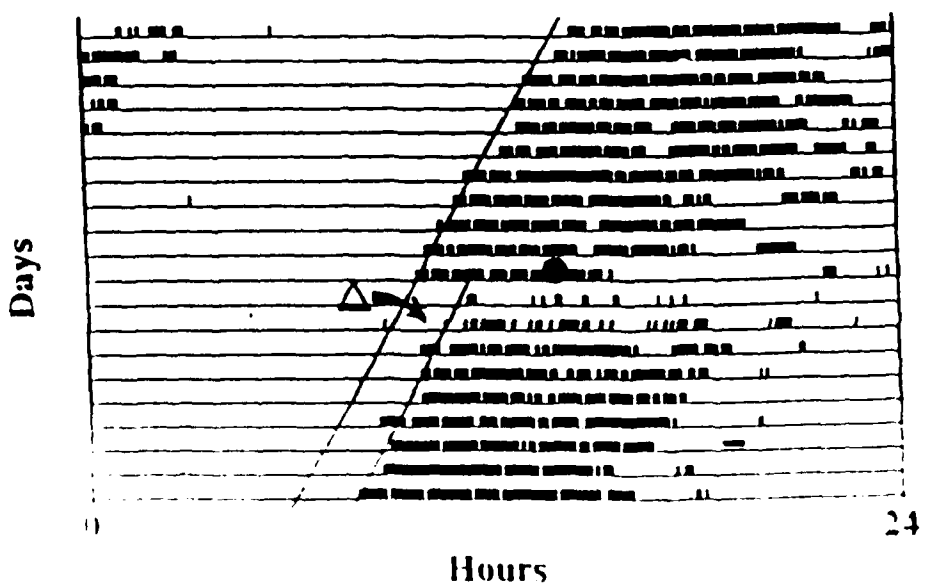
A) +/+

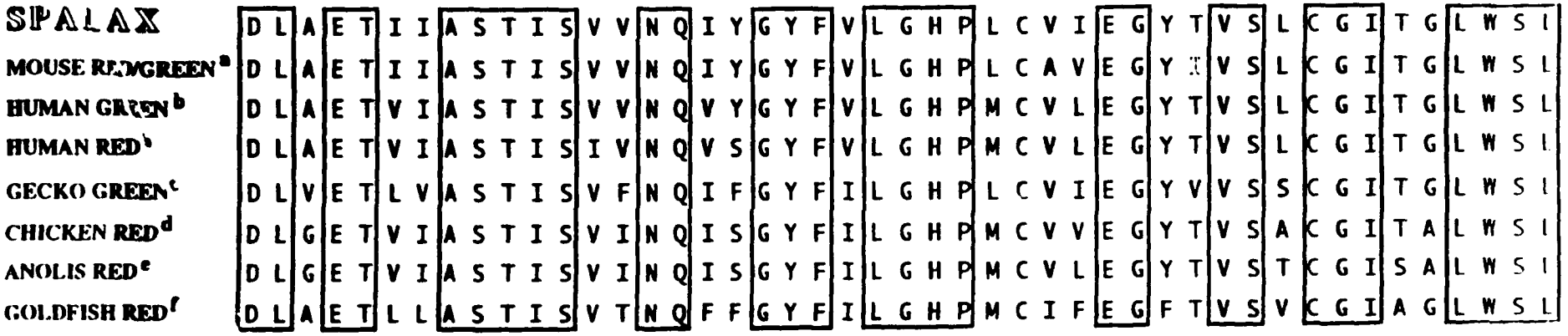


B) *rd/rd*



C) *rds/rds*




SPALAX D L A E T I I A S T I S V V N Q I Y G Y F V L G H P L C V I E G Y T V S L C G I T G L W S L
MOUSE REDGREEN[a] D L A E T I I A S T I S V V N Q I Y G Y F V L G H P L C A V E G Y T V S L C G I T G L W S L
HUMAN GREEN[b] D L A E T V I A S T I S V V N Q V Y G Y F V L G H P M C V L E G Y T V S L C G I T G L W S L
HUMAN RED[b] D L A E T V I A S T I S I V N Q V S G Y F V L G H P M C V L E G Y T V S L C G I T G L W S L
GECKO GREEN[c] D L V E T L V A S T I S V F N Q I F G Y F I L G H P L C V I E G Y V V S S C G I T G L W S L
CHICKEN RED[d] D L G E T V I A S T I S V I N Q I S G Y F I L G H P M C V V E G Y T V S A C G I T A L W S L
ANOLIS RED[e] D L G E T V I A S T I S V I N Q I S G Y F I L G H P M C V L E G Y T V S T C G I S A L W S L
GOLDFISH RED[f] D L A E T L L A S T I S V T N Q F F G Y F I L G H P M C I F E G F T V S V C G I A G L W S L

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Foster, R.G. & Menaker, M. (1993). Circadian photoreception in mammals and other vertebrates. In: L. Wetterberg (Ed.) Vol 62 of *Wenner-Gren International Series, Light and biological rhythms in man*, (pp 73-92). Oxford: Pergamon Press

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Argamaso, S.M., Froehlich, A.C., McCall, M.A., Nevo, E., Provencio, I., Foster, R.G. (1995) Photopigments and circadian systems of vertebrates. *Biophysical Chemistry* **(in press)**

Under Review

Garcia-Fernandez, J.M., Jimenez, A.J., Foster, R.G. (199*) The persistence of cone photoreceptors within the dorsal retina of aged retinally degenerate mice (*rd/rd*): Implications For Circadian Organization. *Neuroscience Letters.*

Jimenez, A.J., Garcia-Fernandez, J.M., Foster, R.G. (199*) Spatio-temporal pattern of degeneration for rods and cones in the retina of rd mice. J. Comp. Neurol.

Manuscripts In Preparation (Submission expected in the next 6 months)

Argamaso S.M., Zenzano, T., Wong, S., & Foster R.G. Expression of rod and cone opsins and circadian responses to light in *rd* and *rds* mice.

Foster R.G., Bovee P.H.M., Provencio I., Kaufmann C., Reisser C., DeGrip W.J. Analysis of the photoreceptor capacity in the developing pineal and retina of rodents: I. The Golden Hamster (*Mesocricetus auratus*).

McCall M., Argamaso, S. M., Stanford, L. R., & Foster, R.G. Rod photoreceptors are not required for circadian responses to light in the mouse.

Provencio I., & Foster R.G. Analysis of circadian photopigments in the retinally degenerate (*rd*) mouse.

Provencio I & Foster R.G. Characterization of the circadian input pathway of the mouse with a neurotropic tract tracing technique.

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Armstrong State College, June 1993

Vanderbildt University. October 1993

University of Reading, UK. November 1993

University of Virginia, Department of Neuroscience. Febuary 1994

Universty of Maryland, February 1994

Oristano, Italy - The Ecology of Vision - Meeting April 1994